

Titration of the binding sites for the oligomycin-sensitivity conferring protein in beef heart submitochondrial particles

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The binding parameters of the oligomycin-sensitivity conferring protein (OSCP) in inside-out particles from beef heart mitochondria have been tested by means of two assays, the oligomycin-sensitive ATP-P_i exchange, and the oligomycin-sensitive ATP hydrolysis. The total number of OSCP binding sites in A particles was equal to 220 pmol/mg particle protein. Each mole of ATPase active site was able to bind 1.1 ± 0.5 mol OSCP with K_d 1.7 nM.

Mitochondrial ATPase

*Oligomycin-sensitivity conferring protein
ATP hydrolysis*

P_i-ATP exchange

1. INTRODUCTION

Oligomycin-sensitivity conferring protein (OSCP) is a small basic protein of M_r close to 20000, belonging to the mitochondrial ATPase F_o-F₁ complex [1-5]. OSCP mediates interactions between the catalytic sector (F₁) and the proton-conducting sector (F_o) of the ATPase complex. The F_o sector, embedded in the mitochondrial membrane, contains a binding site specific for oligomycin. The mediated interaction between F₁ and F_o, for which OSCP is responsible, is probed by the ability of oligomycin, the ligand of F_o, to inhibit the ATPase activity of F₁ [6,7]. Although the recognized mediating function of OSCP makes it implicit that OSCP is a link between F₁ and F_o in the mitochondrial ATPase complex, no direct binding studies on OSCP have appeared. The aim

of this work was to investigate the binding parameters of OSCP by means of two functional assays, the oligomycin-sensitive ATP-P_i exchange and the oligomycin-sensitive ATP hydrolysis, both assays being carried out with inside-out submitochondrial particles.

2. MATERIALS AND METHODS

A-particles were prepared from beef heart mitochondria as in [8]. ASUA particles were prepared by a subsequent treatment of the A particles with Sephadex G-50, urea and ammonia [6,7]. F₁ was prepared from beef heart mitochondria by the procedure in [9]. OSCP was purified as in [4]. Because in our hands this method ended up with a preparation of OSCP slightly contaminated with a small- M_r protein ($M_r \approx 10000$) as shown by SDS gel electrophoresis, an additional step of purification was included, which consisted of a chromatography on Sephadex G-50 (fine) equilibrated in 50 mM Tris-SO₄ (pH 7.5). The purified OSCP in this buffer at 1 mg protein/ml was stored in liquid nitrogen. ATP-³²P_i exchange was measured at 25°C as in [10]. ATPase-oligomycin sensitivity assay was per-

Abbreviations: F₁, beef heart mitochondrial coupling factor; F_o, proton-conducting sector of the ATPase complex, containing the oligomycin binding site; OSCP, oligomycin-sensitivity conferring protein; A particles, submitochondrial particles from beef heart isolated in the presence of ammonia; ASUA particles, A particles successively treated with Sephadex, urea and ammonia

formed at 30°C as detailed in the legend of fig.2. P_i was measured as in [11]. The protein concentration was determined by a dye-binding procedure [12] using bovine serum albumin as standard.

3. RESULTS AND DISCUSSION

3.1. Assay of OSCP by improvement of the efficiency of the ATP- P_i exchange

The A particles used here showed a low ATP- $^{32}P_i$ exchange activity in the absence of OSCP (~ 8 nmol P_i exchanged $\cdot \text{min}^{-1} \cdot \text{mg}$ particle protein $^{-1}$), probably due to partial depletion in F_1 and OSCP and a relatively small number of functional F_1 - F_0 -OSCP assemblies (see also [7]). The experiment illustrated in fig.1 was carried out in the presence of an excess of added F_1 to saturate all available F_0 sites in the particles. Upon addition of increasing concentrations of OSCP, the ATP- $^{32}P_i$ exchange activity increased linearly with

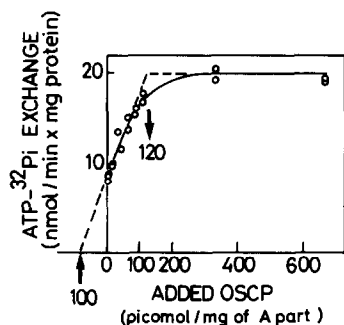


Fig.1. Titration of OSCP binding sites in A particles by the ATP- P_i exchange assay. The assay was performed at 25°C in two steps. In the first step, the F_0 - F_1 -OSCP complex was reconstituted by incubation of A particles (0.25 mg protein) with 0.05 mg of F_1 and increasing concentrations of OSCP for 10 min in a medium containing 30 mM Tris- SO_4 (pH 7.5), 10 mM MgCl_2 and 0.12 mg bovine serum albumin (final vol. 0.45 ml). Then the ATP- $^{32}P_i$ exchange was assayed as follows: to the preincubated particles in the above medium, 0.05 ml of a mixture of 100 mM ATP and 100 mM $^{32}P_i$ were added. The reaction was stopped after 2 min at 25°C by addition of 0.05 ml of 30% perchloric acid at 0°C. After 10 min on ice, the precipitated particles were sedimented by centrifugation; 0.4 ml of the supernatant was withdrawn for extraction of $^{32}P_i$ by a mixture of isobutanol-benzene and determination of the incorporated $^{32}P_i$ in ATP (nmol $P_i \cdot \text{min}^{-1} \cdot \text{mg}$ particle protein $^{-1}$) (see section 2).

respect to the added OSCP, up to a plateau level that corresponded to a 2.5-fold increase with respect to the control ATP- P_i exchange value in the absence of OSCP. A 5-fold stimulation with an excess of OSCP was reported in [2]; clearly, the enhancement factor depends on the degree of depletion of the particles in endogenous F_1 and OSCP, and the membrane leakiness. By extrapolation of the linear portion of the OSCP titration curve to zero activity and determination of the value of the intercept on the abscissa, the amount of endogenous OSCP was evaluated to 100 pmol/mg particle protein. In the same curve (fig.1), the end titration point corresponded to 120 pmol of added OSCP/mg particle protein. The total number of OSCP sites (i.e., the sites occupied by endogenous OSCP and the free sites) was equal to 220 pmol/mg particle protein. This value is close to the amount of oligomycin-sensitive ATPase complexes/mg of submitochondrial particles reported in [13,14]. Taking into account the endogenous OSCP, the concentration of OSCP corresponding to the half-maximum titration in fig.1 was approximated to 80–100 pmol/mg particle protein.

Although the ATP- $^{32}P_i$ exchange assay reflects well the synthetic activity of submitochondrial particles, its main drawback in a reconstituted system is its low efficiency resulting from the leakiness of particles to protons. This may explain the rather high K_d -values for OSCP-binding calculated by this assay (50–100 nM) as compared to those calculated by an ATPase assay described below.

3.2. Assay of OSCP by conferral of oligomycin-sensitivity to membrane-bound F_1 -ATPase

In the present assay, ASUA particles [6] were used as in fig.2A. The assay consisted in the titration of inhibition caused by oligomycin to the reconstituted oligomycin-sensitive ATPase activity in ASUA particles that had been preincubated with a fixed concentration of isolated F_1 and increasing concentrations of OSCP. The amount of ASUA particles was large with respect to added F_1 to offer an excess of binding sites for F_1 ; this explains why addition of large concentrations of OSCP resulted in nearly full inhibition of the ATPase activity. By extrapolation of the OSCP titration curve in the presence of oligomycin to the abscissa, the number of titrated OSCP sites was 50–70 pmol/mg parti-

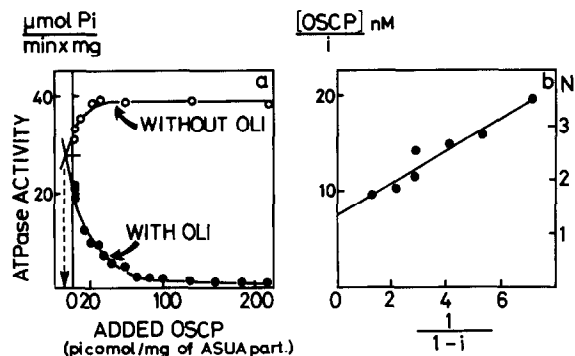


Fig.2. Titration of OSCP binding sites in ASUA particles by reconstitution of the sensitivity of ATPase to oligomycin. (A) The assay was performed at 30°C in two steps. Firstly, increasing amounts of OSCP were preincubated for 10 min with 0.13 mg ASUA particles, 1 μg F_1 and 5 μg oligomycin in a medium consisting of 50 mM Tris- SO_4 , 10 mM KCl and 5 mM $MgSO_4$ (pH 8.0) in 0.45 ml final vol. Secondly, an ATP-regenerating medium (0.05 ml) was added to the above suspension to give the following final concentrations: 5 mM ATP, 5 mM phosphoenolpyruvate (cyclohexylammonium salt), pyruvate kinase 0.04 mg/ml, 5 mM $MgSO_4$, 10 mM KCl and 50 mM Tris- SO_4 (pH 8.0). The ATPase reaction was stopped by addition of 0.10 ml of 50% (w/v) trichloroacetic acid. P_i was measured in 0.05 ml of the deproteinized reaction mixture. ATPase is expressed as μmol P_i formed \cdot min $^{-1}$ \cdot mg added F_1^{-1} . (B) Easson and Stedman plot of data in fig.2A: i refers to the fractional inhibition conferred by oligomycin plus OSCP. The number N refers to the mol OSCP added/mol F_1 .

cle protein. This number is 3–4-times less than that (220 pmol) found in the exchange assay (see section 3.1), simply because in the experiment of fig.2A the amount of added F_1 was limiting with respect to the F_0 sites in the ASUA particles. This explanation was consistent with the results of other assays carried out at different ratios of ASUA particles to F_1 (not shown).

In control particles without oligomycin, a significant stimulation of the ATPase activity by OSCP was observed. The exact nature of this effect is not known; it could be a trivial ionic effect, due to the basic nature of OSCP. It is noteworthy that the δ -subunit of the chloroplastic CF_1 , which has some structural and functional homologies with the mitochondrial OSCP [15] also stimulates the CF_1 -ATPase activity [16].

In the absence of added OSCP, ATPase activity was inhibited by 20% by oligomycin. This small, but significant, inhibition was most likely due to some endogenous OSCP still associated with ASUA particles. The amount of endogenous OSCP was calculated from the intersection of the extrapolated plots of ATPase activities as a function of added OSCP, with and without oligomycin (fig.2A); the intersection corresponded to about 10 pmol of endogenous OSCP/mg of ASUA particle protein; i.e., 14–20% of the titrated OSCP sites in the present experiment (see above). This small fraction of endogenous OSCP remaining in ASUA particles may partly account for the ATPase inhibition in the absence of added OSCP and the presence of oligomycin shown above. It is noteworthy that a non-negligible factor that cannot be readily quantified in the present experiments is probably the lower density of functional F_0 sites for OSCP-binding in ASUA particles as compared to A particles.

A simple way to calculate the OSCP-binding parameters is to assume that OSCP behaves as the limiting factor in the expression of oligomycin sensitivity of the ATPase activity of the F_0 - F_1 complex and to plot the inhibition data by the procedure in [17] for non-competitive inhibition. For this type of representation, the data of fig.2A were used, after correction for the stimulatory effect of OSCP on the ATPase activity of ASUA particles in the absence of oligomycin. The Easson and Stedman equation for ATPase inhibition is:

$$(\text{OSCP})/i = K_d/(1-i) + N$$

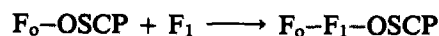
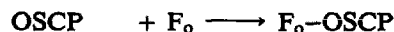
where:

(OSCP)/ i is the reciprocal of the fractional inhibition (i) at a given concentration of OSCP (endogenous plus added OSCP);

N is the number of OSCP binding-sites/active site of membrane-bound F_1 .

From the plot (OSCP)/ i vs $1/(1-i)$, N was found to be equal to 1.3 with an apparent K_d -value of 1.6 nM (fig.2B). The mean average for the N -value from 6 different experiments was 1.1 ± 0.5 mol of sites/mol of F_1 with a K_d of 1.7 ± 1.0 nM. This low K_d -value testifies to the high affinity of OSCP for the F_1 - F_0 complex. The present ATPase assay is probably more reliable than the ATP- P_i exchange assay to assess the affinity of OSCP for

particles, for it does not imply recovery of energy for a synthetic reaction like ATP-P_i exchange. These experiments do not allow us to determine the sequence of events in the formation of an F₁-F_o-OSCP complex. However, the finding that, in the absence of added OSCP, F₁ exhibited some oligomycin sensitivity, indicates that the F_o sector of a small fraction of ASUA particles still contains some bound OSCP. This suggests that a possible sequence of reaction, among others, is:



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